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Stabilization of liposomal membranes by thermozeaxanthins: carotenoid-glucoside esters

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Abstract

Thermozeaxanthins (TZS) are novel carotenoid-glucoside esters existing in the cell membranes of the thermophilic bacterium, *Thermus thermophilus*. The effect of TZS on membrane permeability was studied by measuring the leakage of the fluorescent dye from calcein-entrapped large unilamellar liposomes (LUVs). The LUVs were composed of a small portion (0.2–1.0 mol%) of TZS and phosphatidylcholine (PC) of various length and saturation degree of hydrocarbon chains. Incorporation of TZS in egg PC LUVs stabilized the liposomes in the temperature range from 30 to 80°C, as only 2.6% of the entrapped calcein leaked out in contrast to 10% release from the egg PC liposomes without TZS. LUVs composed of dipalmitoylphosphatidylcholine (DPPC) or dioleoylphosphatidylcholine (DOPC) were stabilized by the incorporation of TZS at a temperature below 30°C. Inclusion of TZS in LUVs composed of dimyristoylphosphatidylcholine, whose hydrocarbon chains are shorter than both DPPC and DOPC, did not stabilize the liposomes. About 90% of the entrapped dye was lost indicating defects of the liposomal membranes. Matching of the lipid bilayer thickness with the molecular length of TZS in the bilayers is discussed.

Keywords: Thermozeaxanthin; Zeaxanthin; Carotenoid; Liposome; Stabilization; Calcein; *Thermus thermophilus*

1. Introduction

Carotenoids such as zeaxanthin, the terminally hydroxylated carotenoids found in many bacteria, can be inserted into lipid bilayers as rivets [1], and brace together the two leaflets of the bilayers to reinforce them [2–5]. Incorporation of polar carotenoids in lipid bilayer usually results in a decrease in membrane fluidity and an increase in membrane stability. The stabilization effect of carotenoids has been studied using liposome model membranes composed of phospholipids [2,3,5] or ether lipids extracted from archea [4]. The thermophilic bacterium *Thermus thermophilus* can live and grow in hot springs, and most

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Thermus strains have been found to produce carotenoids [6].

Yokoyama et al. [7,8] recently isolated thermozeaxanthins (TZS) from the lipid extracts of *T. thermophilus*, and identified its chemical structure. As illustrated in Fig. 1, TZS are esters of zeaxanthin monoglucoside consisting of zeaxanthin, glucose, and branched fatty acids. In membranes, the rigid conjugated hydrocarbon chains of TZS are supposed to be located in the hydrophobic core of lipid bilayers while the glucose moieties are anchored in the hydrophilic headgroup region, and the branched fatty acids moieties curl back into the hydrophobic region like a 'hook' [7], to reinforce the membranes. TZS are exclusive to *T. thermophilus* as the main constituents of the carotenoid fraction [8]. The amphiphilic feature of TZS and its existence in the membranes of this bacterium led us to postulate that thermozeaxanthins may play an ordering effect in membranes similar to that of dihydroxylated carotenoids to stabilize the membranes [7]. However, there are few experimental data to further support this hypothesis. In the present work, we studied the effect of TZS on membrane permeabilities at a wide temperature range using liposomes composed of synthetic phospholipidylcholine (PC) of different hydrocarbon chains or natural PC with heterogeneous hydrocarbon chains. Matching of the lipid bilayer with

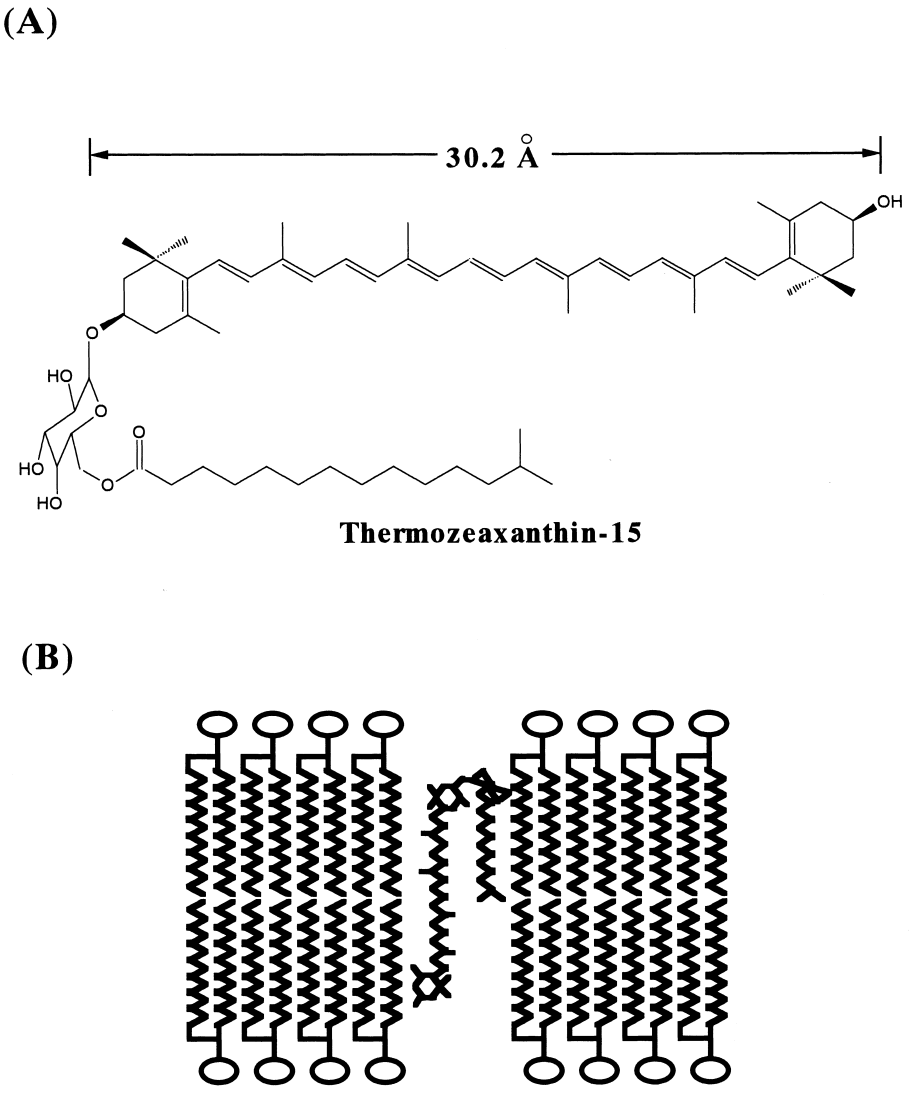

Fig. 1. Structure of thermozeaxanthins (TZS) (A) and schematic representation of TZS incorporated in lipid bilayer (B).

the TZS molecules is discussed in relation to the stability effect.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (egg PC, purity 95%), dioleoylphosphatidylcholine (DOPC), and dimyristoylphosphatidylcholine (DMPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA); phospholipids extracted from *Escherichia coli* containing 50% phosphatidylethanolamine (denoted *E. coli* PE) and dipalmitoylphosphatidylcholine (DPPC) were from Sigma (St. Louis, MO, USA). 3,3′-Bis[*N*,*N*-di(carboxymethyl)-aminomethyl] fluorescein (calcein) was purchased from Dojindo Laboratories (Kumamoto, Japan). Sephacryl S-300 was from Amersham Pharmacia Biotech (Uppsala, Sweden). Other chemicals were analytical grade.

2.2. Isolation of thermozeaxanthins

TZS were extracted from the carotenoid-overproducing recombinant *T. thermophilus* Cop101(pCOP1) and its chemical structure was elucidated as described before [6,7]. The TZS used in this report are a mixture of esters of zeaxanthin monoglucoside, namely zeaxanthin monoglucoside esterified by two kinds of fatty acids of carbon 13 and 15, respectively.

2.3. Preparation of liposomes

Large unilamellar vesicles (LUVs) containing TZS were prepared by the extrusion method as described before [9,10]. Briefly, egg PC, DPPC, DOPC or DMPC were mixed with TZS (molar ratio 0.2% or 1.0%) in chloroform and dried to a lipid film by nitrogen flushing and rotary evaporation under a high vacuum. The dried lipid film was dispersed in 2 ml of 100 mM calcein (pH 7.5) to form MLVs (lipid concentration was 10 mg/ml). The MLV suspension was then frozen in liquid nitrogen and thawed in a water-bath at a temperature range from 25 to 50°C (see below) for five cycles, followed by passing 10 times through two stacked polycarbonate filters of 100 nm pore size (Nuclepore, Costar, Cambridge, MA, USA) at 25°C for egg PC, 30°C for DMPC, 50°C for DPPC on a high-pressure vesicle extruder equipped with a temperature controller (Lipex Biomembrane, Vancouver, BC, Canada). Unentrapped calcein was removed by gel filtration on a Sephacryl S-300 column (2×35 cm), which had been equilibrated with 50 mM Tris-HCl (pH 7.5) (buffer T). Calcein-free LUVs were prepared by the same procedures as described above except that buffer T was used to form MLVs instead of the 100 mM calcein solution.

The mean size of the liposomes was analyzed by dynamic light scattering (DLS) essentially as described in [10], and was 90 nm ± 29.1 for egg PC, 107 nm ± 21.7 for *E. coli* PE, 94 nm ± 31.5 for DPPC, and 81 nm ± 21.8 for DOPC LUVs. The liposomes were in buffer T in the absence of free calcein. A DLS analyzer equipped with argon laser (488 nm) (DLS-6000 AS, Otsuka Electronics, Osaka, Japan) was used with the scattering angle set at 90°C and temperature at 25°C.

2.4. Fluorescence measurement

Leakage of calcein from the liposomes was determined by fluorescence measurement with an excitation at 488 nm and emission at 517 nm. A fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) equipped with a temperature controller was used and the change of the fluorescence was determined by a time-scan mode. The calcein-loaded liposomes were diluted 1000-fold with buffer T in a cuvette and kept for a few minutes at a desired temperature; release of the calcein from the liposomal interiors was monitored as a function of time. The percentage of the released calcein was calculated as follows:

$$\% \text{ release} = (F' - F_0)/(F_t - F_0) \times 100.$$

F' is the fluorescence intensity determined under various temperatures or time periods, F_0 and F_t are the initial and total fluorescence intensity defined as before and after addition of Triton X-100 to a final concentration of 0.03% (w/v) at 4°C. For measuring the extent of calcein leakage from liposomes by the treatment of detergent, 20 μl of 0.3% Triton X-100 was added to the diluted liposomes in a cuvette and the change in fluorescence was monitored as the con-

centration of Triton X-100 in the suspension was increased from 48 to 200 μM. Fluorescence intensity increased stepwise by the successive addition of the detergent and then reached a plateau region at a detergent concentration above 0.3% (w/v). The difference in fluorescence intensity between the initial level and each step was divided by the maximal value in the plateau region to calculate the percentage of calcein leakage.

3. Results

3.1. Effect of TZS on stability of liposomes with natural lipids at different temperatures

The influence of TZS on the stability of liposomal lipid bilayers was studied by measuring the extent of calcein release from LUVs. Fig. 2A showed that less than 4% of the entrapped dye leaked out in the temperature range 60–80°C from the egg PC LUVs containing TZS, compared to more than 8% loss of the calcein for the LUVs composed of egg PC alone in the same temperature range. The difference in the leakage between the LUVs with and those without TZS was significant at the temperatures above 40°C. An increase in the amount of TZS from 0.2 mol% to 1.0 mol% resulted in a decrease in the leakage of the calcein (Fig. 2A). This means that the liposomes with TZS incorporated became more stable than the LUVs without the TZS. The TZS molecules were well matched with egg PC molecules in the liposome membranes resulting in increased thermostability of the liposomes. LUVs composed of *E. coli* PE and TZS were also found to be more stable than the liposomes with *E. coli* PE alone at temperatures below 40°C (Fig. 2B). However, the stabilization effect of TZS was less clear in the *E. coli* PE LUVs than in the egg PC LUVs, particularly at high temperature (Fig. 2B). This may be attributed to the difference in their lipid composition.

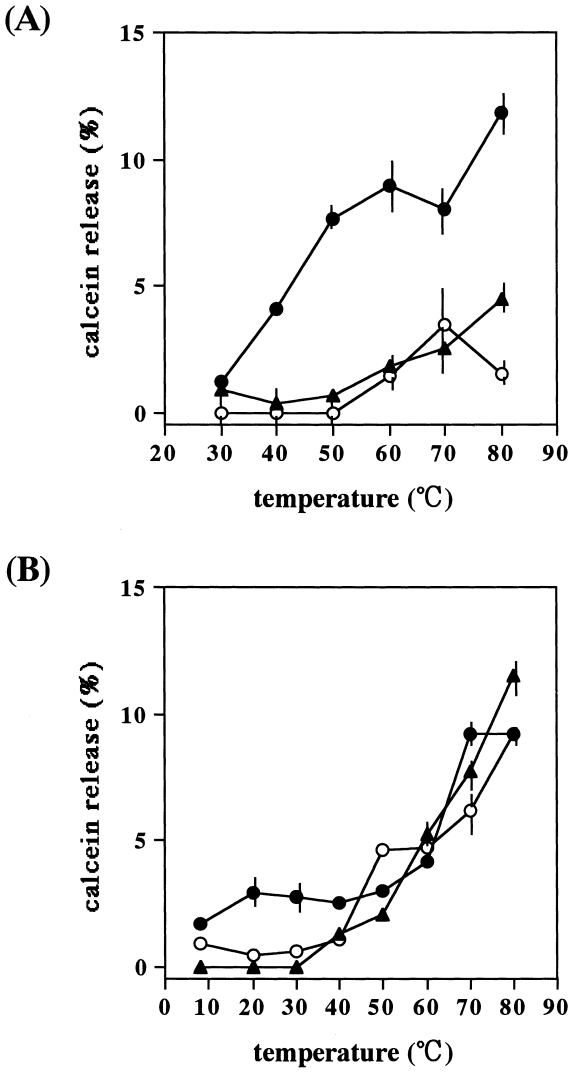

Fig. 2. Percentage of released calcein from LUVs composed of egg PC (A) and those of *E. coli* PE (B) as a function of temperature. Filled circles, filled triangles and open circles represent the data with LUVs containing 0, 0.2 and 1.0 mol% TZS, respectively. Calcein-entrapped liposomes were incubated for 30 min at each temperature to estimate the leakage of calcein as described in Section 2. Bars show the standard deviations on data points ($n > 3$).

3.2. Effect of TZS on stability of liposomes with synthetic lipids

Apart from the natural phospholipids, several kinds of synthetic PC of various lengths and saturation degree of its alkyl chains, i.e., DMPC (14:0) with 14 carbons in the alkyl chains, DPPC (16:0) with the alkyl chains two carbons longer than that of DMPC, and DOPC (18:1) with two carbons longer and one double bond of chains compared to that of DPPC, were also used to study the effect of the hydrocarbon chains of PC on the membranes containing TZS. TZS showed no apparent effect on calcein leakage from DPPC LUVs as shown in Fig. 3A

although DPPC LUVs containing TZS showed a slightly lower leakage (2.03 ± 0.18%) than the control LUVs without TZS (2.70 ± 0.12%) at 20°C. This conflicts with the effect of zeaxanthin on the dye release from liposomes which depressed and broadened the transition temperature of DPPC or DMPC liposomes [2,3,11]. As shown in Fig. 3B for DOPC LUVs, incorporation of TZS in the liposomes had a small stabilization effect at temperatures below 40°C, but had an destabilization effect at 50°C and

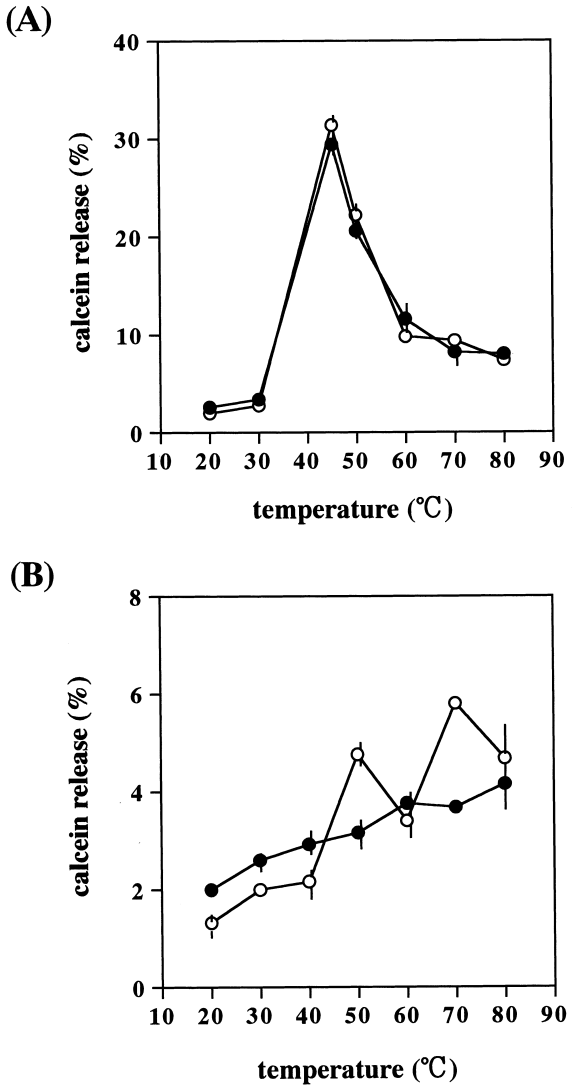

Fig. 3. Percentage of released calcein from LUVs composed of DPPC (A) and those of DOPC (B) as a function of temperature. Filled circles and open circles represent the data with LUVs containing 0 and 1.0 mol% TZS, respectively. Other conditions were the same as in Fig. 2.

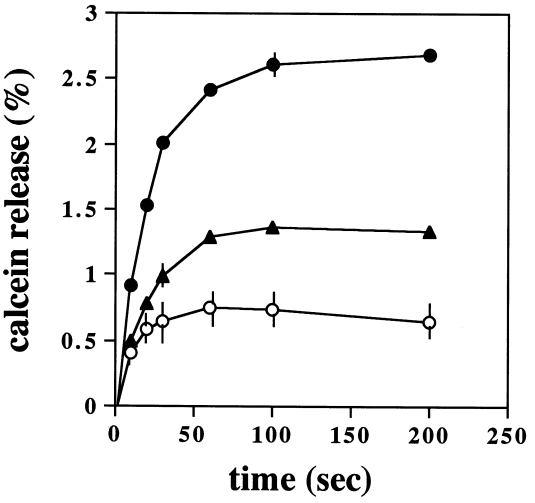

Fig. 4. Percentage of released calcein from LUVs composed of DPPC as a function of time at room temperature (25°C). Filled circles, filled triangles and open circles represent the data with LUVs containing 0, 0.2 and 1.0 mol% TZS, respectively.

70°C. We do not have a good explanation for this discrepancy now. In contrast to both the DPPC and DOPC LUVs, the DMPC liposomes were far more leaky, up to 90% dye was lost by introducing TZS into their membranes even at the low temperatures studied (data not shown).

The membrane permeability of DPPC liposomes was measured by the time-dependent release of calcein entrapped in the liposomes with or without TZS. It was found that addition of TZS to DPPC liposomes suppressed the time-dependent leakage of calcein from the calcein-entrapped liposomes at room temperature (Fig. 4). A lower release (0.75%) of dye was observed for DPPC LUVs with 1.0 mol% of TZS at room temperature while the release of calcein from liposomes without TZS was about 2.7%. This stabilization effect was observed in a wide pH range from 5.0 to 9.0 (data not shown), and the liposomes with 1.0 mol% of TZS were more stable than those with 0.2 mol%. These results are consistent with the observation that the thermozeaxanthins weakly stabilized DPPC liposomes at low temperatures in the temperature-dependent measurement (Fig. 3A).

3.3. *Effect of TZS on liposomes in the presence of detergent*

In addition to the thermostability of the TZS-con-

taining liposomes, the stabilization effect of the TZS on membrane permeability was investigated in the presence of detergent at various pHs. TZS did not stabilize liposome membranes composed of synthetic PC (e.g., DPPC) upon the action of detergent at pH 7.5 and also in a wide pH range from 5.0 to 9.0 (data not shown). Thermostability and stability against detergent seem be based on different mechanisms.

4. Discussion

As schematically illustrated in Fig. 1 and [7], the orientation of TZS molecules in lipid bilayers greatly depends on the thickness of the hydrophobic core of lipid bilayers and the kinds of lipids which formed the lipid bilayers. The thickness of the lipid bilayer seems to be important both for the conjugated rigid hydrocarbon chains and for the fatty acid tails of thermozeaxanthins, because the alkyl chains of lipids not only supplied the hydrophobic core to interact with the rigid molecule of thermozeaxanthins, but had to fill up the space not occupied by fatty acid tails of thermozeaxanthins. This implies that TZS required more subtle adjustment to resolve the packing problem between the carotenoids and lipids.

Thermozeaxanthins bear rigid rod-shaped conjugated hydrocarbon chains, the distance between the oxygen atoms in the two hydroxyl groups at the ends of the long hydrocarbon chain was 30.2 Å, similar to the distance of zeaxanthin [1]. The thicknesses of lipids bilayers investigated have been estimated to be 27.9 Å (DMPC), 29.3 Å (DPPC), and 30.6 Å (DSPC) [2]. The thickness of DOPC was evaluated to be about 30 Å (deduced from the thickness of DSPC which has the same carbon number in the alkyl chain as DOPC). Mostly, egg PC was a mixture of C16 and C18 saturated alkyl chains at C-1, and C18 unsaturated alkyl chain at C-2 (according to the manufacturer's specification described in the Avanti catalogue). Therefore the thickness of egg PC bilayer was estimated to be 30 Å as a mean value of DPPC and DSPC.

In the case of zeaxanthin, which has only a single rigid hydrocarbon chain with two hydroxyl groups at positions 3 and 3′, the orientation depended on the thickness of the lipid and the saturation of the alkyl chains. It could be inserted almost perpendicularly in the DPPC and DSPC membranes because the molecular length of zeaxanthin was almost equal to the thickness of the DPPC or DSPC hydrophobic core [2]. But it had a tendency to tilt with respect to the normal plane of the DMPC membrane because the thickness of DMPC was not large enough to accommodate the molecular length of zeaxanthin [2,12]. Zeaxanthin also showed a much better incorporation in egg PC membrane but did not show a measurable effect [13]. The effect of TZS and that of zeaxanthin showed some differences because the glucose esters are special moieties in TZS in addition to the rigid molecular bar in zeaxanthin. Both a corresponding thickness and alkyl chains of lipids were necessary for orientation of TZS into lipid membranes. Egg PC with various hydrocarbon chains seems to provide an appropriate thickness of lipid bilayers for the conjugated rod-shaped molecule of TZS and a better environment to resolve the packing problem caused by the fatty acid esters in TZS. It was not surprising that the carotenoids investigated can stabilize the egg PC liposomes at a wide range of temperatures.

TZS showed a good match with DPPC and DOPC membranes as zeaxanthin did with DPPC and DSPC membranes [2]. TZS in DMPC membranes tilted with respect to the plane of the membrane, as zeaxanthin did in DMPC liposomes. The shorter alkyl chains of DMPC lipids could not alleviate the packing problem caused by the tilted fatty acid tails of TZS. As a result the well matched lipid bilayers were disturbed by the introduction of TZS and resulted in calcein release from those liposomes. In addition, the rigid molecule of carotenoids preferred to interact with extended alkyl chains from the membrane surface to the center of lipids. The *trans* conformation of alkyl chains in lipids would be matched better with this rigid molecule. When the membranes of lipids become more fluid at higher temperature, the alkyl chains like to adopt a *gauche* conformation rather than an all-*trans* conformation. This was probably the reason why the effects of zeaxanthin [3] and TZS on synthetic lipids diminished at temperatures above the phase transition temperature.

Fig. 3A shows that maximal leakage of calcein was observed around the phase transition temperature (T_m). This is consistent with other reports that permeation of certain molecules was enhanced appreciably around T_m [14,15]

The liposomes containing TZS were easily perturbed by the addition of Triton X-100. When TZS intercalated into the membranes, the headgroups in lipids were separated by the polar moieties of TZS, the packing of headgroups was weakened and produced additional possibilities for 'binding' of Triton X-100 into lipids. We used natural and synthetic phospholipids to analyze the matching problem in this report. We can conclude from all the results that generally speaking, liposomes of natural lipids like egg PC showed a clearer stabilizing effect by TZS in a wide range of temperatures than the liposomes of synthetic lipids. We suppose that TZS could find suitable hydrocarbon chains to be matched well with the hydrophobic surface of TZS in the case of natural lipids containing heterogeneous hydrocarbon chains, however single species of lipids with defined fatty acid chain length could not match well with both the rigid rod-shaped carotenoid moiety and fatty acid moiety of TZS. Our preparation of TZS used in this research contained two kinds of fatty acids of carbon lengths 13 and 15 as described in Section 2. Synthetic TZS will be more useful to focus on the matching between lipids and TZS in future investigation.

It is known that cholesterol, hopanoid carotenoids and other membrane-spanning lipids are used as stabilizers of lipid bilayer membranes in eukarya, bacteria and archaea, respectively [16]. The latter two kinds were membrane-spanning molecules to reinforce the lipid bilayer by bracing together the two leaflets of the bilayers as rivets whereas the former stabilized one leaflet by itself. With TZS as one of the main end products of carotenoid biosynthesis in *T. thermophilus* TZS probably does reinforce the cell membranes of bacteria. This feature of TZS enables the bacteria to survive at high temperatures. It should also be pointed out that only a small amount (0.2 and 1.0 mol%) of TZS was used in our work in comparison with zeaxanthin. It was reported that maximal 'solubility' of zeaxanthin in DMPC was 8.5% [1]. A higher proportion of TZS to lipids will show bigger stabilizing effect although we have not tested this because of limitations in the amount of extracted TZS. We firstly showed that TZS has a stabilizing effect on liposomes of phospholipids. These molecules will be useful as natural, nontoxic membrane stabilizers for many applications such as drug delivery systems in future investigations.

Acknowledgements

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